

1-1-2013

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BOZALIOĞLU, SEMA; KILIÇ, NEDRET; YILDIRIM, ZUHAL; BAŞTÜRK, BİLKAY; and KURUKAHVECİOĞLU, OSMAN (2013) "The relationship between HLA-G levels and oxidative stress parameters in patients with breast cancer," *Turkish Journal of Medical Sciences*: Vol. 43: No. 6, Article 2. <https://doi.org/10.3906/sag-1206-4>

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The relationship between HLA-G levels and oxidative stress parameters in patients with breast cancer

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Received: 01.06.2012 • Accepted: 01.01.2013 • Published Online: 02.10.2013 • Printed: 01.11.2013

Aim: To investigate serum HLA-G levels and its correlation with oxidative stress and antioxidant parameters in breast cancer patients.

Materials and methods: The patient group consisted of 25 individuals with breast cancer who were operated on in the General Surgery Department of Gazi University's Faculty of Medicine. Thirty-one individuals who had no history of any kind of cancer, allergic diseases, diabetes, or rheumatic and immunological disease constituted the control group.

Results: Results showed that malondialdehyde (MDA), advanced oxidation protein products (AOPPs), and HLA-G levels in the patient group were statistically and significantly high compared to the control group ($P < 0.001$). While vitamin C levels were significantly lower in the patient group ($P < 0.05$), an insignificant difference in glutathione peroxidase (GPx) and total sulfhydryl group (R-SH) levels in both groups was observed ($P > 0.05$). Serum superoxide dismutase (SOD) levels in the patient group were significantly low compared to the control group ($P < 0.001$). In the control group, statistically significant positive correlations among HLA-G, MDA ($r = 0.580$, $P = 0.001$), and AOPP ($r = 0.569$, $P = 0.01$) were observed. In the control group, a significant positive correlation in MDA and AOPP levels ($r = 0.482$, $P = 0.006$) was observed.

Conclusion: Results of the present study indicated that there was a relationship between the oxidative stress and the deficiency of antioxidant defenses as lipid peroxidation, protein oxidation, and HLA-G expression increased.

Key words: Oxidative stress, antioxidants, breast cancer, HLA-G

1. Introduction

Breast cancer is the most frequently seen type of cancer in the world. According to the results of a 2006 European study, it ranked first among women, with a morbidity rate of 28.9%. Breast cancer was responsible for 17.6% of cancer-related deaths (1,2). It ranks first in cancer-related deaths in every developed country except for Japan (3).

The molecular mechanisms underlying the development of cancer have not been fully clarified yet. It is thought that genetic damage (mostly cumulative in nature) leads to the activation of protooncogenes and to deactivation of tumor-suppressor genes, which triggers breast cancer. This manifests as uncontrolled cell proliferation or the death of the wrong cells and apoptosis (4). However, the fact that the families of some individuals also have a cancer story specific to the area or regions where they live indicates that cancer can also be developed through genetic mutations (5). Besides this, reactive oxygen species are thought to play a

role in cancer development. Most of the risk factors for breast cancer are related to overexposure to increased estrogen. It is believed that estrogen stimulates the proliferation of the breast cells, thereby increasing the chance of proliferation of the cells that carry the mutated genes that may cause cancer (4). Direct DNA damage can be incurred in the target tissue without reacting with some estradiol estrone metabolites (6). Cancer cells are characterized by a reduction in the control of growth or no control at all, an invasion of the local tissues, and metastasis (7).

Oxidative stress plays a role in the etiopathogenesis of many benign or malignant diseases, such as diabetes mellitus, cardiovascular diseases, and cancer (8,9). Oxidative stress is a natural process in all living forms that need oxygen, and it is made up of several steps. Biological systems incorporate some specific mechanisms that control this stress. A lack of control mechanisms causes oxidative damage (10,11).

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An increase in oxidative stress disturbs the balance between the oxidant and antioxidant system. It also causes molecular oxygen to produce some reactive oxygen metabolites, such as superoxide radicals, hydrogen peroxide, hydroxide, and peroxide radicals. The free radical and reactive oxygen metabolites that are formed lead to oxidative damage in DNA and lipids, which are the fundamental structural molecules of the body (12–17).

Insufficient and suppressed response of the immune system allows tumors to develop. The subgroups of HLA molecules, whose relationship with several diseases has been shown, are defined in the immune response (18,19). HLA-G is not a typical class I molecule and the recent research has shown that this molecule is likely to play a role in the development of immunological tolerance. In the research that has been carried out, HLA-G has been detected in the peripheral blood and acidic liquids around the tumor and in the tissues of cancer patients. It is known that the HLA-G molecule is expressed by a number of tumor tissues and that with the effect of the tumor microsurrounding, the tumor escapes from the immune system's response. HLA-G molecules suppress natural killer cells and T lymphocytes and allow an increase in the secretion of cytokines (20–24).

The purpose of this study was to investigate whether there is a relation between the levels of HLA-G and reactive oxygen products caused by oxidative stress in the patient group with breast cancer and in the healthy control group.

2. Materials and methods

2.1. Subjects

In this study, the patient group consisted of 25 patients that had been operated on for breast cancer in the Department of General Surgery in the Faculty of Medicine at Gazi University. The control group consisted of 31 healthy individuals aged between 35 and 40 who did not have any history of cancer, allergic diseases, diabetes, or rheumatologic or immunologic diseases. HLA-G analysis was carried out in the plasma samples taken from patients and those in the control group, while the levels of glutathione peroxidase (GPx), superoxide dismutase (SOD), malonyldialdehyde (MDA), advanced oxidation protein products (AOPPs), vitamin C, and total sulfhydryl groups (R-SH) were measured in serums.

Blood samples were drawn from the patients and the controls after overnight fasting, then centrifuged as soon as possible at $2000 \times g$ for 10 min at 4 °C. Serum and plasma samples were stored at -70 °C until the analyses were carried out.

AOPP levels were measured by a spectrophotometric method in the presence of potassium iodide at 340 nm and calibrated with chloramine-T solutions (25). AOPP levels were expressed in micromoles chloramine-T equivalents

per liter. Lipid peroxidation (in terms of MDA) was estimated using the thiobarbituric acid reactive substances (TBARS) test as described previously (26). Briefly, TBARS formation was quantified using 1,1,3,3-tetraethoxypropane as the standard, and the absorbances of the TBARS were read at 532 nm using a Shimadzu UV 1601 spectrophotometer (Shimadzu, Tokyo, Japan).

Reduced glutathione (GSH) levels were expressed as total sulfhydryl group (R-SH) levels (27). First, 0.5 mL of each sample was mixed with 1 mL of a solution containing 100 mM Tris-HCl (pH 8.2), 1% sodium dodecyl sulfate, and 2 mM ethylene diamino tetra acetic acid. The mixture was incubated for 5 min at 25 °C and centrifuged to remove any precipitate. After that, 0.3 mM 5,5-dithiobis-(2-nitrobenzoic acid) was then added to each reaction volume and incubated for 15 min at 37 °C. The absorbance of each sample was determined at 412 nm. The R-SH levels were calculated assuming a molar extinction coefficient of $13.000 \text{ mol}^{-1} \text{ cm}^{-1}$ at 412 nm. The SOD activity measurements were carried out by inhibiting the SOD activity via nitroblue tetrazolium reduction. Xanthine-xanthine oxidase was used as a superoxide generator, and 1 IU was defined as the quantity of SOD required to produce 50% inhibition (28).

The GPx activity was determined spectrophotometrically as described in the literature (29). The reaction mixture, containing 50 mM phosphate buffer (pH 7.4), 7.7 units GSH reductase, 5 mM GSH, and crude extract, was preincubated for 5 min at 37 °C. Thereafter, 20 μL of nicotinamide adenine dinucleotide phosphate in reduced form (NADPH) (0.3 mM) solution was added and the hydroperoxide-independent consumption of NADPH was monitored for about 5 min. The overall reaction was started by adding 20 μL of prewarmed hydroperoxide solution (0.025 mM). A decrease in absorption at 340 nm was monitored.

The total level of ascorbate was determined by the modified Roe and Kuether method (30). Serum samples were added to trichloroacetic acid solution and centrifuged at $3.000 \times g$ for 10 min. 2,4-Dinitrophenylhydrazine-thiourea-copper sulfate reagent was added to the serum sample tubes. The contents of each tube were mixed, capped with Parafilm, and placed in a water bath at 37 °C for 4 h. The tubes were removed and cooled in ice water. Ice-cold 65% sulfuric acid solution was added and mixed thoroughly. The mixture was allowed to stand at room temperature for 30 min, and absorbance was measured using a Shimadzu UV 1601 spectrophotometer at 515 and 520 nm, respectively. The lower limit of detection for vitamin C was 0.05 $\mu\text{mol/L}$. The determination of HLA-G was done through enzyme-linked immunosorbent assay (ELISA), one of the serological tests in researching antibodies or antigens, using Bio Vonder Human sHLA-G ELISA RD-1504.

2.2. Statistical analysis

SPSS 15.0 for Windows was used for statistical analysis. In detecting HLA-G, ROC analysis was used to find the threshold level to diagnose the disease. The Student t-test was used to find the differences in averages regarding the variables examined in the control and the patient group. The degree of the relationship between variables was calculated through Pearson correlation analysis. $P < 0.05$ was taken as significant.

3. Results

In this study, we measured AOPP, MDA, SOD, GPx, R-SH, vitamin C, and HLA-G levels in the samples drawn from patients with breast cancer at phases 2 and 3, and we examined the possible correlations among them. The mean values of the investigated parameters are given in the Table.

It has been observed that MDA, AOPP, and HLA-G levels are significantly higher in patients with breast cancer than those in the healthy control group ($P < 0.001$; Figures 1, 2, and 3, respectively).

The levels of vitamin C in patients with cancer are significantly lower than in the control group ($P < 0.05$; Figure 4), while there are no notable statistical differences in GPx and R-SH levels ($P > 0.05$; Figure 5 and Figure 6, respectively). Serum SOD levels have been found to be significantly lower in patients with breast cancer than in the control group ($P < 0.001$; Figure 7).

When the correlations of the parameters in both the patient and control groups were examined, no notable statistical correlations in the patient group were observed. However, in the control group, statistically significant positive correlations among HLA-G, MDA ($r = 0.580$, $P = 0.001$), and AOPP ($r = 0.569$, $P = 0.01$) were observed.

Table. The mean values of the investigated parameters in the patient and the control groups (mean \pm SD).

	Patient group (n = 25)	Control group (n = 31)	P
AOPP ($\mu\text{mol/L}$)	228.65 \pm 127.00	138.31 \pm 63.64	$P < 0.001$
MDA (nmol/mL)	11.42 \pm 2.22	5.20 \pm 1.63	$P < 0.001$
GPx (U/mL)	35.32 \pm 32.79	32.14 \pm 16.95	$P > 0.05$
Vitamin C ($\mu\text{mol/L}$)	1.66 \pm 0.83	2.31 \pm 1.09	$P < 0.05$
R-SH (nmol/mL)	278.47 \pm 122.80	253.22 \pm 43.93	$P > 0.05$
SOD (U/mL)	5.70 \pm 2.01	13.00 \pm 2.99	$P < 0.001$
HLA-G (U/mL)	80.65 \pm 46.82	37.97 \pm 21.47	$P < 0.001$

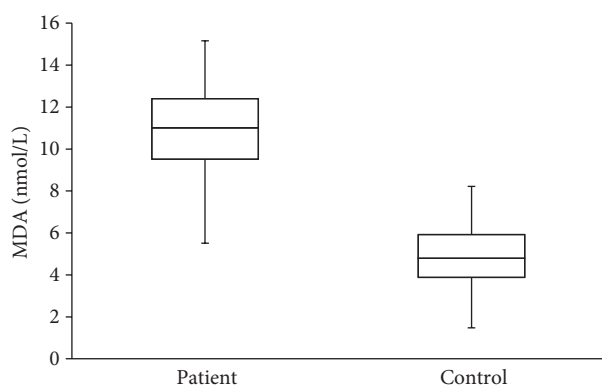


Figure 1. Mean MDA levels in the patient and the control groups.

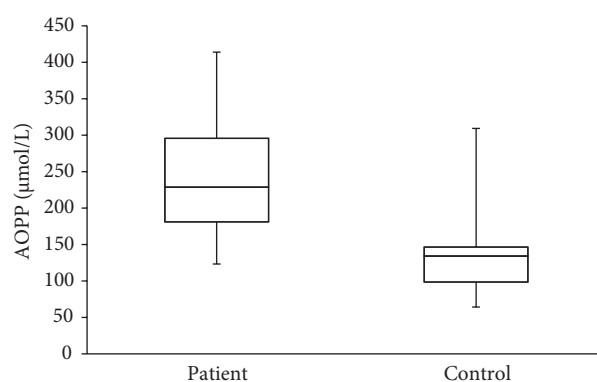


Figure 2. Mean AOPP levels in the patient and the control groups.

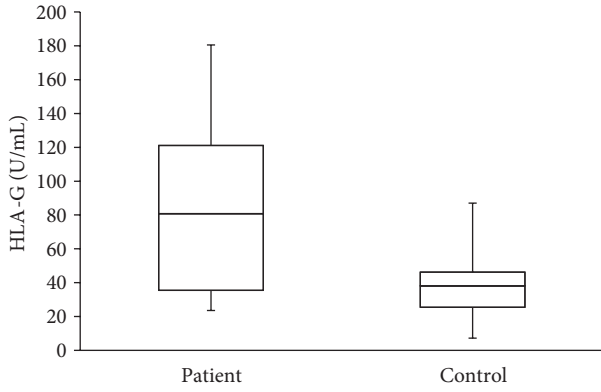


Figure 3. Mean HLA-G levels in the patient and the control groups.

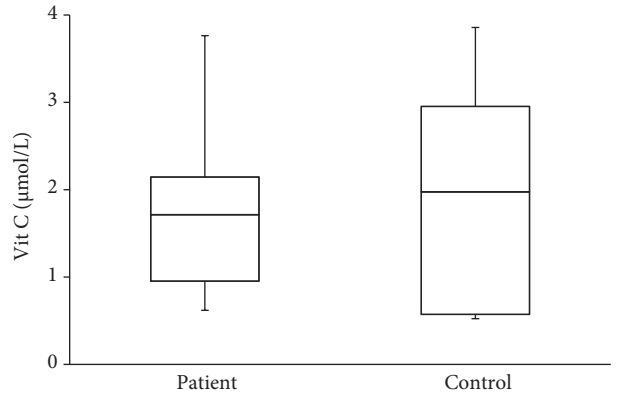


Figure 4. Mean vitamin C levels in the patient and the control groups.

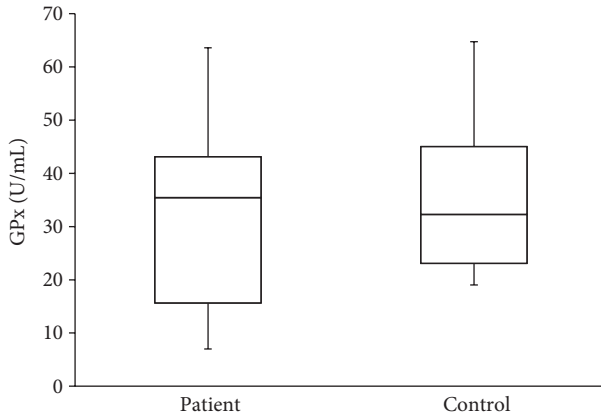


Figure 5. Mean GPx levels in the patient and the control groups.

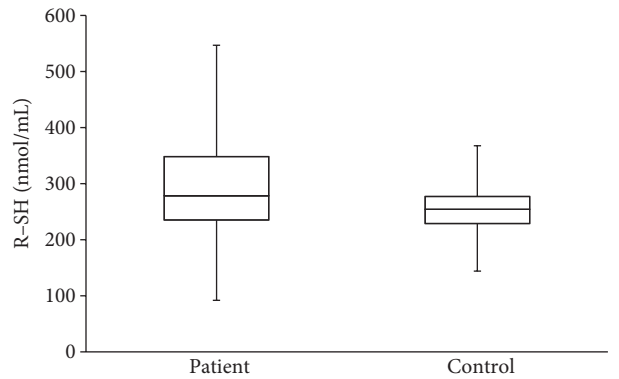


Figure 6. Mean R-SH levels in the patient and the control groups.

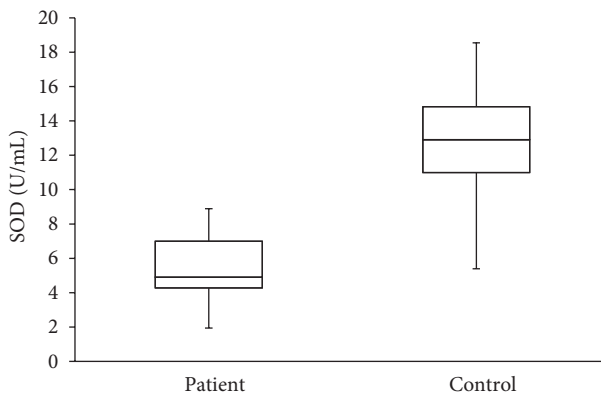


Figure 7. Mean SOD levels in the patient and the control groups.

While there were significant positive correlations between MDA and AOPP levels in the control group ($r = 0.482$, $P = 0.006$), there were no significant correlations among the other parameters measured.

When the patient group is considered in terms of metastasis, there were significant differences in measured parameters between those who had lymph nodes and those who did not ($P > 0.05$). Significant statistical differences were also observed between those who had angiolymphatic invasion and those who did not ($P > 0.05$).

4. Discussion

Epidemiologic and clinical studies show that free oxygen radicals play an important role in cancer pathogenesis. The damage done to the breast epithelium may cause fibroblast proliferation, epithelial hyperplasia, cell differentiation, and breast cancer (31–33). A number of findings from lipid peroxidation studies indicate that in several types of cancers, lipid peroxidation increases in solid tumors (34,35). Although there are many studies suggesting that the levels of MDA are high in patients with breast cancer (36,37), there are also studies where lower levels of MDA have been detected (38,39). Additionally, in our studies,

in accordance with the studies where high levels of MDA in plasma or cancerous tissues have been measured, significantly high levels of MDA have been observed.

Reactive oxygen types and oxidative stress products have been known to cause protein oxidation products in protein structures as well as membrane lipids through covalent modifications (40). Badid et al. (41) detected significantly high levels of protein carbonyl products in their studies with breast cancer patients. In our study, it was also observed that in accordance with the literature, there are significantly high proportions of AOPP in patients with breast cancer. When the correlation of the parameters measured in patient and control groups was examined, a statistically remarkable correlation in the parameters measured in the patient group was observed. While there is a significant positive correlation between the MDA and AOPP levels in the control group, there is no significant correlation among the other parameters measured.

Some studies show that oxidative stress causes upregulation in antioxidant enzymes and that antioxidant enzyme expression increases in cancers (41,42). The clinical studies supporting this certify that SOD enzyme activity in breast cancer patients is higher in accordance with the phase of the disease (32,38). On the other hand, in the research carried out by Gibanananda et al. (43), it was reported that SOD and GPx activities increased, while catalase (CAT) activity decreased.

Sinha et al. (44), however, found that the SOD and CAT activities were significantly low in breast cancer patients aged 30–50 whose lipid peroxidation levels in plasma and tissues were high. Similarly, Kasapovic et al. (45) detected that depending on age, lipid peroxidation was high in breast cancer patients of different ages, whereas the level of antioxidants was low. Researchers attributed the low SOD activity observed in cancer patients to the decrease in enzyme synthesis due to aging. In previous studies, researchers mostly pointed out the increase in the antioxidant enzyme activities. However, also in this study, the findings related to the antioxidant levels support the recent studies showing that these enzyme activities are low. Furthermore, a difference has been observed in SOD activities in breast cancer patients with different phases. However, this enzyme activity was significantly low when compared to that of healthy control groups. This difference can be explained by the decrease in enzyme synthesis capacity depending on age; the healthy control group was aged 35–40, while the patient group was aged 45–65.

GSH is an important peptide inside the cell. It takes part in many functions, ranging from antioxidant protection to regulating cell proliferation. GPx, glutathione S-transferase, free radicals, and peroxides play a central role in the defense mechanisms against many xenobiotics and

carcinogens (46). The serum levels of R-SH were examined in this study. When compared to the control group, an insignificant increase in R-SH was detected in patients with breast cancer. When considered in connection with SOD, this finding has led us to the idea that R-SH is not a primary antioxidant in breast cancer patients. Another important function of glutathione in an organism is to reconvert vitamin C radicals into vitamin C formed during the regeneration of tocopheroxyl radicals (47). In some studies, it has been shown that there is a considerable increase in GPx activity, which is the first phase of the enzyme defense against H_2O_2 and other hydroperoxides in tumors (41,43). High antioxidant enzyme levels and low GSH levels were detected in breast cancer patients by Yeh et al. (47). Although no significant differences have been observed in total thiol levels and GPx enzyme activities, significantly low levels of vitamin C have been measured in breast cancer patients compared to the control group. Ascorbic acid, together with glutathione, is known to be the most important antioxidant in liquid phase (48). Tocopheroxyl contributes to preventing carcinogenesis by stimulating the immune system (49). In one study carried out consisting of patients with breast, gastrointestinal system, and lung cancer, Torun et al. (50) showed that lipid peroxidation increased while vitamin C levels remained significantly low.

In the studies conducted by Gibanananda et al. (43) in 54 breast cancer patients in different phases, it was shown that vitamin C levels in the patients, and especially in phase 1 and phase 4, are significantly low compared to those in the healthy control group. Prospective studies come up with different results regarding the relationship between vitamin C and breast cancer (51). In a similar study carried out by Nissen et al. (52) it was demonstrated that the incidence of cancer increases with the intake of vitamin C.

It has been shown *in vitro* that vitamin C acts both as an antioxidant and a prooxidant. However, *in vivo* studies showed that vitamin C has mostly antioxidant effects (52). It has been shown that HLA-G molecules, which are associated with many diseases, play a role in pregnancy and organ transplantations. The HLA-G expression in trophoblasts plays a role in protecting the fetus against the mother's NK cells during pregnancy. In this period, it is known that microframe and secreted cytokines and chemokines also have a role (20–22). Findings showed that the effect of the microenvironment has a crucial role in HLA-G expression. Findings also support that there is a negative feedback balance between the immune system and HLA-G. As long as the immune system responses are maintained within the expected balance, it is likely that HLA-G expressions will not increase.

Recently, a number of studies investigating the relationship between HLA-G and malign diseases have been published. In the study done by Palmisano et al. (23) it was found that HLA class I molecules totally disappeared in the low-phase tumor tissue (G1, G2) with minimal stromal contamination. It was also reported that no HLA-G expressions were detected. In another study, extremely high HLA-G expressions were found in breast cancer tissue where there was a significant inflammation caused by immune cell activity. HLA-G expressions have been attributed to this inflammation (53). However, RT-PCR analysis shows that there is an HLA-G activation at a transcriptional phase in breast tumors (23). However, there has been no HLA-G expression in breast cancer cell series kept in a prolonged period of culture environment.

In the results of this study, there was a significant statistical difference between the HLA-G levels of the patient group and the healthy group. Statistically significant positive correlations between HLA-G and MDA and AOPPs were observed in this study. The high level of plasma HLA-G has made us think that the immune response to the tumor remained insufficient and that the response was suppressed by HLA-G through certain mechanisms. HLA-G expression varies depending on the factors that are caused by microframe, tumor, or tumor cells (54).

The cytokines and interferon secreted by the immune cells located around the tumor have an accelerating effect on the HLA-G expressions, especially from IL-10 tumor cells, epithelial cells, monocytes, and macrophages (55). IL-10, as is known, is a kind of cytokine ensuring Th2-type polarization. The effect of both are considered

to be responsible for increasing immune-suppressing pathophysiology in the development of cancer (54). Tumor cells can express or secrete HLA-G molecules on the surface of the cell. In the studies carried out, it has been shown that HLA-G molecules can pass to an NK cell activated with a cell membrane from a tumor cell that expresses HLA-G.

In our study, when the patient group are considered regarding metastasis, there was a significant difference in the parameters in patients with lymph metastasis and those without metastasis. When patients who have angiolympathic invasion and those who do not are considered, no significant statistical difference has been observed (56–58).

In light of these findings, it has been concluded that the high levels of HLA-G in the examined breast cancer patients at phases 2 and 3 might have caused the immune system to be suppressed, and thus the immune system response to the tumor remained insufficient. As it is also shown in our findings, among the factors that change the HLA-G expression in breast cancer, the increase in the reactive oxygen species and an insufficient amount of antioxidants might be effective. However, in HLA-G expression, there is a need for further research into factors that appear according to the stage of the cancer within a microframe, as well as factors that are caused by the cells themselves.

Acknowledgements

This study was supported by Gazi University Research Foundation Project No. TF.01 / 2007 - 102. Endorsement for the study was granted by the Gazi University Ethical Committee.

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